CHAPTER 6

IN VITRO STUDIES ON

SPACE RADIATION-INDUCED

DELAYED GENETIC RESPONSES:

SHIELDING EFFECTS

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ABSTRACT

Understanding the radiation risks involved in spaceflight is of considerable importance, especially with the long-term occupation of ISS and the planned crewed exploration missions. Several independent causes may contribute to the overall risk to astronauts exposed to the complex space environment, such as exposure to GCR as well as SPEs. Protons and high-Z energetic particles comprise the GCR spectrum and may exert considerable biological effects even at low fluence. There are also considerable uncertainties associated with secondary particle effects (e.g. HZE fragments, neutrons etc.). The interaction of protons and high-LET particles with biological materials at all levels of biological organization needs to be investigated fully in order to establish a scientific basis for risk assessment. The results of these types of investigation will foster the development of appropriately directed countermeasures.

Since the early 1990s, a number of high profile studies have shown that, as well as acute effects present in cells a few divisions after irradiation, longer-term changes occur in the surviving cells leading to pronounced genomic/chromosomal instability. There are genetic components in mouse and man that determine the relative sensitivity of their cells to radiation-induced genomic instability and ultimately their cancer risk. The genomic instability phenotype may be explained as manifestations of transmissible genomic aberrations that occur at higher frequencies than would be expected from the statistics of individual particle hits to surviving cell nuclei, and may be transmitted from an irradiated cell to a non-irradiated cell by a bystander effect mechanism. However, little is known about either the underlying mechanisms or the *in vivo* consequences of such induced phenotype(s).

In this study, we compared the biological responses to proton irradiation presented to the target cells as a monoenergetic beam of particles of complex composition delivered to cells outside or inside a tissue phantom head placed in the United States EVA space suit helmet. Measurements of chromosome aberrations, apoptosis, and the induction of key proteins were made in bone marrow from CBA/CaJ and C57BL/6 mice at early and late times post exposure to radiation at 0, 0.5, 1 and 2 Gy while inside or outside of the helmet. The data showed that proton irradiation induced transmissible chromosomal/genomic instability in haematopoietic stem cells in both strains of mice under both irradiation conditions and especially at low doses. Although differences were noted between the mouse strains in the degree and kinetics of transforming growth factor-β1 and tumor necrosis factor-α secretion, there were no significant differences observed in the level of the induced instability under either radiation condition, or for both strains of mice. Consequently, when normalized to physical dose, the monoenergetic proton field present inside the helmet-protected phantom produced equivalent biological responses, when compared to unshielded cells, as measured by the induction of delayed genetic effects in *murine* haematopoietic stem cells.

6.1 INTRODUCTION

The assembly of ISS is expected to require 1000 hours of EVA during a period of high solar activity. Exposure to GCR and SPEs are a major health risk to humans. Protons and high Z, energetic particles comprise the GCR spectrum and may exert considerable biological effects even at low fluence. During the construction of ISS, the astronauts could be exposed to substantial levels of protons and electrons from SPEs and trapped radiation belts [Space Rad Health Vol1 (1) 2001]. To estimate the protective properties and characterize radiation transport processes they provided, the U.S. and Russian suits were irradiated with protons and electrons at LLUMC facilities in California.

Recent studies have shown that, in addition to acute effects of radiation that occur within a few cell divisions, longer-term changes occur in the surviving cells, leading to pronounced chromosomal instability (Kadhim et al., 1992; Kadhim et al., 1995; Kadhim et al., 2001). However, and perhaps more importantly, there are genetic components in mouse and man that determine the relative sensitivity of their cells to radiation-induced genomic instability and ultimately their cancer risk. Additionally, untargeted genetic lesions occur wherein the induced "mutation rate" at specific loci are higher than can be attributed to initial or direct radiation-induced DNA damage. Transmitted genetic instability may in part be due to a bystander effect mechanism. Despite many high-profile studies, little is known about either the underlying mechanisms or the in vivo consequences of such induced phenotype(s).

In this study, we conducted in vitro biological experiments using murine haematopoietic stem cells. Cytogenetic aberrations, apoptosis, and cytokine production were assessed in bone marrow cultures derived from CBA/CaJ and C57BL/6 mice harvested at early and late times post exposure to proton beam radiation while inside or outside a human tissue phantom placed inside the United States EMU.

6.2 MATERIALS AND METHODS

6.2.1 Source of Proton Radiation

Proton beam irradiations were performed using 250 MeV protons from the LLUPTF synchrotron accelerator. Protons were delivered from the accelerator in 0.3 s pulses every 2.2 s (Moyers, 1999). Calibration of the dose received by the cells was performed using an N.I.S.T.-traceable Markus™ parallel plate ionization chamber in a polystyrene phantom set to mimic the cell culture set-up. The ICRU 59 calibration method (*ICRU*, Report **59**, 1998) was used to convert the ionization signal to dose. The dose ranged from 0 to 2 Gy delivered at a dose rate of approximately 0.35 Gy/minute.

6.2.2 Setup of Phantom Head and EMU Helmet

To compare the responses of cells in a relatively pure proton beam to responses under simulated conditions inside the body of an astronaut wearing space suit protection, we placed polypropylene vials of cells in 1) the entrance region of a monoenergetic beam or 2) inside a tissue phantom head covered by the U.S. EMU suit helmet. The two setups were calibrated with an Exradin model T1 thimble ionization chamber placed at the vial location that was adjusted to beam isocenter.

The unshielded configuration consisted of cells placed in polypropylene cryovials located at isocenter behind a 1-cm buildup layer of polystyrene. Delivered physical doses were: 0, 50.3 or 100.3 and 200.1 cGy. For the shielded condition, the vials of cells were placed in a row of machined holes located near the center of the head section of the phantom (average of 90 mm from the "skin" surface at the level of the orbits). The phantom was placed inside the U.S. EMU helmet with sun visor up and facing the oncoming beam. The arrangement of target cells in the phantom head within the helmet and the non-shielded set of cells are shown in the four-panel composite labeled **Figure 6-1**.

The phantom is a custom sliced model # RAN100C (ser. # 731) RANDO® Man Phantom. (The Phantom Laboratory, P.O. Box 511, Salem, NY) It is a radiologically accurate phantom representing a 50% anthropometric standard male determined from an Air Force survey renormalized to civilian populations. The material of construction is based on an isocyanate rubber whose density and effective atomic composition are based on the ICRP standard Man for muscle equivalent soft tissue. A natural human skeleton is embedded in the phantom.

6.2.3 Source of Haemopoietic Bone Marrow Stem Cells/In Vitro Culture Conditions

Bone marrow stem cells from two inbred strains of mice, known to be sensitive (CBA) or resistant (C57) to high-LET type of radiation-induced delayed expression of chromosomal/genomic instability (Watson et al. 1997), were selected for use in this study. Male C57 (n =10) and CBA (n=10) mice were purchased from Jackson Laboratories (Bar Harbor, MA), housed and maintained according to specific regulations specified in our approved animal protocol in accordance with the current animal welfare act. From each strain of 10- to 12-week-old animals, we obtained femoral bone marrow cells by removal of the epiphysis and metaphysis at the proximal and distal ends of the femurs and flushing the cells out of the marrow cavity by distal insertion of a 21-gauge needle attached to a syringe containing complete medium (Alpha-MEM, supplemented with 10% fetal calf serum and 1mM glutamine).

The isolated cells were counted and two sets of single-cell suspensions were placed in 2-ml vials and irradiated at 0, 0.5, 1.0 and 2.0 Gy placed either inside the phantom and helmet, or outside the helmet. Immediately after irradiation, haemopoietic stem cell cultures were established by transferring cells to T-75 cm^2 flasks containing 30 ml of medium supplemented with 25% pretested horse serum, antibiotics, and pretested conditioned medium from the AF1.19T and L929 cell lines as sources of colony stimulating activity (Kadhim et al. 1992). Cells were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air for a total of 7 days.

Forty-eight hours post-irradiation, 20 ml of the culture were removed from each flask to assess initial genetic damage (Kadhim *et al*. 1992). The cells were used for cytogenetic analysis, and a battery of immunocytochemical measurements [apoptosis (annexin V) and BrdU incorporation into enzymatically induced DNA cleavage] (Green *et.al.* 2001). The supernatant were stored frozen at –70°C and later tested for cytokines released into the supernatant (Gridley *et al.* 1996).

The remaining cultures received 10 ml of fresh medium, supplemented with serum and growth factors (as described above) and incubated an additional 5 days. At the end of the 7-day period (12-15 cell divisions) the

progeny of the surviving cells were assayed for delayed effects of radiation exposure by repeating the cytogenetic, immunocytochemical, and cytokine measurements.

6.2.4 Cytogenetic Analysis

Coded chromosome preparations of various cell populations were made by accumulating metaphases in the presence of 0.02 μg/ml Colcemid for 2h, followed by treatment with 0.5% (w/v) potassium chloride and fixation in methanol:acetic acid (3:1 v/v). Fixed cells were spread on slides, air dried, aged for 1 week at room temperature, and stained with Giemsa. To determine the frequency of karyotypic abnormalities, evident as induced and transmissible chromosomal instability in the progeny of surviving cells, except those samples with low mitotic index, a maximum of 100 well spread metaphases per time point and treatment condition were analyzed.

6.2.5 Apoptosis-DNA Damage

6.2.5.1 Annexin V Binding Assay

When cells are damaged and begin to undergo apoptosis, there is an early event wherein phosphatidylserine is displaced from the inner surface to the outer surface of the plasma membrane (Majno & Joris, 1995). This early event can be detected by the binding of annexin V. To assess the proportion of cells in the irradiated and control cultures that were in early stages of apoptosis, we used an FITC-annexin V labeling procedure (PharMingen, San Diego, CA), modified as described in Green et al. 2001. Bone marrow cells from in vitro cultures were incubated with FITC-conjugated annexin V for 45 min at room temperature. The cells were then centrifuged and washed with wash buffer, fixed in -20°C 70% ethanol for 15 min, rehydrated in PBS and counter-stained with propidium iodide (PI) and RNAse for 30 min. The cells were centrifuged and washed in wash buffer before spreading onto microscope slides. The cells were protected with permafluor (Fisher Scientific) and covered with glass coverslips. The slides were dried flat in the dark and scanned on the laser scanning cytometer (CompuCyte, Cambridge, MA).

All of the cell nuclei were labeled red (PI), which allowed them to be located and counted by the laser scanning cytometer. The PI measurements were placed on the y-axis as integrated fluorescence. The x-axis was set to measure green (FITC-annexin V) integral fluorescence. The optimized protocol and display settings were confirmed visually by the microscope camera and stored for use in subsequent analysis of all cells analyzed in these experiments. The average number of cells scanned per slide was 1500+/-200.

6.2.5.2 DNA Damage-BrdU Incorporation

Bone marrow in vitro cultured cells were harvested at early (48 hours) and late (7 days) times post irradiation to measure DNA strand breaks using terminal deoxynucleotidyl-transferase (TdT) mediated fluorescent (FITC)-conjugated BrdU incorporation into free 3' ends of nucleic acids. This methodology does not distinguish single from double strand breaks in the DNA molecules (Li, 1995). The procedure has been previously described in Green et al. 2001. Briefly, cells were fixed in -20°C 70% ethanol for 15 min. The fixed cells were then rehydrated in PBS for 5 min and incubated with a mixture of TdT, reaction buffer, and FITC-BrdU provided with the kit. Cells were

incubated with the DNA labeling mixture overnight at room temperature (22-24°C), washed and counter-stained for 30 min with PI/RNAse, washed, and placed on microscope slides protected with permafluor.

FITC-BrdU incorporated into DNA strand breaks were quantified using the laser scanning cytometer. To measure DNA damage, scanning parameters were adjusted by signal intensity to create a contour of the PI nuclear label. To quantify the cells that were both red and green, the gating parameters were set to contour on red (PI) (y-axis) and to sum (integrate) the green fluorescence intensity (FITC-BrdU) (x-axis). Green fluorescent intensity reflected a quantitative measure of DNA damage, as exposed 3' ends, in the irradiated and control cells. Optimized protocol and display settings were stored and used for all samples in these experiments. An average of 1,000+/-200 cells were scanned per side.

6.2.6 Analysis for Cytokines

Quantification of *murine* tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-2, and granulocyte/ macrophage-colony stimulating factor (GM-CSF) in bone marrow cell culture supernatants were performed using enzyme-linked immunosorbent assays (ELISA) (Quantikine^{TM} kits, R & D Systems, Minneapolis, MN). These tests are based on the multiple antibody sandwich technique that results in a color reaction and were performed according to the manufacturer's instructions. Supernatants were first centrifuged to remove nonadherent (floating) cells or cell debris. The absorbance in each well was measured in an automated plate reader (Model Fluorite 1000, Dynex Technologies, Inc., Chantilly, VA) equipped with Revelation software version 3.0 and set at appropriate wavelength. Concentration of each cytokine in the test samples was obtained from the respective standard curve. Quantification of transforming growth factor-β1 (TGF-β1) was performed using an ELISA technique (R & D Systems), similar to those for the cytokines described above. However, before testing for TGF-β1, test samples, standards, and control media were activated by acidification with 1N HCl for 1 hr and subsequently neutralized with 1N NaOH to pH 7.0-7.4. Thus, this assay measured the total TGF-β1 (i.e., latent plus biologically active forms). Assay sensitivities were: 5.1 pg/ml (TNF-α), 3 pg/ml (IL-1β), 3 pg/ml (IL-2), 1 pg/ml (GM-CSF), and 7 pg/ml (TGF-β1). The number of viable bone marrow cells in the cultures had been determined at harvest by manual counting in the presence of trypan blue. The pg/ml of each cytokine measured was normalized to pg/10^5 cells.

6.3 RESULTS

6.3.1 Cytogenetic Analysis

Bone marrow stem cells from CBA and C57 mice irradiated while shielded or unshielded from proton radiation were assessed for chromosomal aberrations at early and late times post-irradiation. The early time corresponded to 1-2 cell divisions and the late to 12-15 cells division equivalents, respectively. Aberrations were detected in all cultures and at all doses whether they were placed inside or outside the shielded location. Examples of the types of aberrations typically seen in metaphase chromosome spreads are shown in **Figure 6-2**. Graphs of the total aberrations for the two strains of mice are shown in **Figure 6-3** and specifics on the types of aberrations scored are compiled in **Table 6-1**.

The CBA mouse marrow cells scored at the early time point showed that the unshielded marrow had a reduced frequency of aberration compared to the shielded cells. For the C57 early sample, there was no significant difference between the shielded or unshielded samples. Both strains showed a tendency for the cells exposed to the highest dose of radiation (2 Gy) to have a lower relative level of aberrations than either the 0.5 or 1 Gy irradiated samples (**Table 6-1**, **Figure 6-3**). In the late time point (12-15 population doublings) samples, the proportion of chromosomally aberrant cells from both strains of mice was highly significant compared to the non-irradiated control group (see p values in **Table 6-1**). This finding was consistent for the lower doses and under both irradiation conditions, with no apparent dose response. The expression of instability determined by chromatid type of aberrations was declining in the 2 Gy proton-irradiated groups under both conditions, the level however remained elevated relative to the control cell levels (p values are in **Table 6-1**).

6.3.2 Apoptosis-DNA Damage

The sum of annexin V binding (early apoptosis) and BrdU incorporation (late apoptosis) was used to reflect the total level of cell death occurring in the bone marrow cultures at the two selected harvest times. The values for these two assays are listed in **Table 6-2**. Generally, the strand break assay (BrdU) yielded higher levels for all samples than did the binding of annexin V. Thus, the majority of cells undergoing radiation-induced cell death were in the later stages of apoptosis/necrosis at the early harvest, which was reflected by DNA fragmentation. At the early harvest post-irradiation, the highest level of apoptosis occurred in the CBA cultures at 1 Gy, whereas the C57 cultures had their highest level of apoptosis occurring at 0.5 Gy. There was no radiation dose response in either the shielded or unshielded cells, but the samples that were shielded had a generally higher level of total apoptosis (**Table 6-2**, **Figure 6-4**).

The apoptotic measurements taken from the cultures harvested at the late time point lacked a dose response similar to that seen in the early harvested cultures. However, the major difference between the early and late harvests were the dose at which the peak cell damage occurred. The highest level of apoptosis occurred in the CBA cultures at 0.5 Gy, whereas the C57 cultures had their highest level of apoptosis occurring at 1 Gy. The CBA late harvest, like the early harvest, had generally higher levels of apoptosis occurring in the shielded cultures. C57 mouse marrow had higher levels of apoptosis occurring, but there was no significant difference between shielded or unshielded samples ($p > 0.5$) (**Table 6-2**, **Figure 6-4**).

6.3.3 Cytokine Production/Release in Irradiated and Control Culture Supernatants

The supernatant harvested from the early and late, shielded and non-shielded cultures were tested for various cytokines; the only significant production was for TGF-β1 and TNF-α (**Table 6-3**). TGF-β1 concentrations increased dramatically in supernatants of cells from both strains of mice compared to non-irradiated cultures under certain conditions. With CBA cells, the greatest increase in TGF-β1 was at 7 days after a shielded dose of 0.5 Gy (23-fold higher than 0 Gy), whereas the peak level in C57 cells occurred at 48 hr after the unshielded dose of 1 Gy (16.5-fold higher than 0 Gy) (**Figure 6-5**). Changes in TNF-α expression were also observed (**Table 6-3**, **Figure 6-6**), but to a much lesser degree than with TGF-β1. With CBA cells, the greatest increase was at 7 days following a dose of 0.5 Gy

(2.0-fold higher than 0 Gy); the highest levels in supernatants from C57 cells occurred at 7 days after a 2-Gy shielded dose of radiation (3.6-fold higher than 0 Gy). IL-1, IL-2, and GM-CSF were below detectable levels in supernatants from non-irradiated controls, as well as those from irradiated cells, regardless of shielding (data not shown).

6.4 DISCUSSION

The cytogenetic investigations demonstrate that proton irradiation can induce transmissible chromosomal/ genomic instability in hematopoietic stem cells in both strains of mice under both irradiation conditions, especially at low doses. Previous studies found that the CBA mice were sensitive to the induction of genomic instability in response to high-LET radiation, whereas the C57 mice were relatively resistant (Watson et al. 1997). The major cytogenetic aberrations (chromatid breaks, chromosome fragments) were consistent with the transmission of chromosomal instability. Moreover, the frequency of induced instability was considerably greater than the frequency of induced mutations at specific loci which are characterized by a higher ratio of chromatid to chromosome-type aberrations (Kadhim et al. 1992). In this study, at the times measured, we have demonstrated cytogenetic abnormalities, and detected significant apoptosis in the progeny of stem cell cultures from both strains of mice and under both conditions post-irradiation. There was an inverse relationship between aberrations and apoptosis; this was especially so for the C57 mouse bone marrow cultures (see **Tables 6-1** and **6-2**). These findings have significant implications, in that instability induced per irradiated cell(s) was higher than the number of initial cells at risk, and appears to be exaggerated at the lower doses under both radiation conditions. In one-half of the samples, there was a trend wherein the increased level of TGF-β? measured was coincident with the increased level of cytogenetic aberrations.

The selection of cytokines for assay was based on evidence that they are radiation-inducible, associated with malignant transformation, and/or are important regulators of hematopoiesis (Fortunel et al. 2000; Hallahan 1996). Studies with cytotoxic drugs that affect primarily proliferating cells have demonstrated that TGF-β plays a protective role in the bone marrow by inhibiting stem/progenitor cell cycling, an effect that is later reversible (Bottinger et al. 1997). In the present study, the peak level of TGF-β1 produced by irradiated CBA cells was approximately 3.5-fold higher than that produced by irradiated C57 cells (**Table 6-3**). It is tempting to speculate that the high TGF-β1 produced by the CBA strain may have protected progenitors in which subtle radiation-induced mutations existed (e.g. inactivation of a gene involved in growth regulation), but were not detectable by the methods used. Indeed, it has been proposed that genomic instability and mutations leading to defects in TGF-β signaling may work in concert to accelerate tumor progression in multistage carcinogenesis (Glick et al. 1999). If this is proven true, it may account at least partly for the high susceptibility of CBA/Ca mice to radiation-induced acute myeloid leukemia. Our observation of somewhat lower levels of apoptosis in the CBA cultures, suggesting that these cells are less prone to die as a result of irradiation compared to C57 cells, is consistent with this premise. However, the well-documented transforming potential of TGF-β1 and the variable responses of different bone marrow cell populations to the factor should also be considered.

TNF-α is a cytokine that can induce oxygen radical production, DNA damage, and apoptosis (Gupta et al. 1992; Rath & Aggarwal 1999), and has, like TGF-β, been associated with genetic instability (Rosselli et al, 1994). Studies also indicate that TNF-α can augment the effects of radiation on a variety of cell types (Gridey 1996;

Hallahan 1996). The presented data show that peak TNF-α levels occurred at late harvest for both strains of mice, but no consistent patterns or correlations with the other measurements were identified.

In conclusion, the present study has demonstrated the induction of transmissible chromosome/genomic instability in *murine* haematopoietic stem cells after proton irradiation whether shielded or unshielded, that were most pronounced at the lower doses implemented. Consistent with previous observations, the frequency of induced instability in the present study was considerably greater than the frequency of conventional chromosome aberrations or gene mutations, which persisted as a result of the direct effect of ionizing radiation. The absence of significant differences in the level of instability between the shielded and unshielded radiation conditions suggest that at equal physical dose there was no quantitative difference in the biological response between the complex radiation environment produced by shielding components and the relatively pure proton beam. The differences in TGF-β1 and, to a much lesser extent, TNF-α expression between the two strains of mice warrant further investigation regarding the role that these cytokines may play in radiation-induced leukemogenesis.

6.5 ACKNOWLEDGMENTS

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